IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 09/297,092
Applicant : Michael PAULISTA et al
Filed : May 18, 1999
TC/A.U. : 1636
Examiner : Sumesh Kaushal

Docket No. : 2923-115
Customer No. : 6449
Confirmation No. : 9258

DECLARATION UNDER 37 C.F.R. 1.132

Director of the United States Patent
and Trademark Office
P.O. Box 1450
Alexandria, Virginia 22313-1450

I, Dr. Jens Pohl, hereby declare as follows:

1. I am the same Jens Pohl listed as one of the inventors on the above-referenced patent application.

2. I have carefully reviewed the Office Action dated October 1, 2003. Experiments were carried out under my direction and control which show that a fragment containing only the cysteine-knot core of MP52 is able to bind to its receptor and exhibits the desired cartilage and/or bond inducing activity. The experiments were conducted as follows:

As described in the patent application, MP52 is a growth and differentiation factor belonging to the bone morphogenetic protein (BMP) subfamily of TGF-ß like molecules. Since most of these proteins are potent morphogenetic inducers causing e.g. osteoblast differentiation, osteoinduction and chondrogenesis, BMPs are effective tools for bone fracture healing, cartilage repair and other procedures where protein-induced morphogenesis is desirable. Like all members of the TGF-ß family, BMPs such as MP52 are naturally synthesized as larger precursor molecules and subsequently processed by proteases to form mature proteins of approximately 110 up to 140 amino acids. The amino acid sequence of the mature part of all BMPs is highly

conserved and comprises seven cysteine amino acid residues at the carboxyterminal position. Six of these cysteines are involved in the formation of intrachain disulfide bonds which create a rigid „cystine-knot" structure, whereas the seventh cysteine residue is needed to form bioactive dimeric molecules via a single interchain bond. The seven conserved cysteine residues which form the three dimensional scaffold structure (cystine-knot) are important for the structure and activity of BMPs whereas the N-terminal part is dispensable for protein structure and function. The correct folding of the „cystine-knot" structure of BMPs is required for specific interactions with BMPR-I and BMPR-II receptors located on the cell surface. The BMP cystine-knot exhibits a hydrophobic "wrist" epitope which shows high affinity to the extracellular ligand binding domain of the receptor. The protein receptor interaction results in phosphorylation of BMPR-II which finally transduces the signal by additional phosphorylation of „second messenger" Smad proteins. The activated Smads heterodimerize, enter the nucleus and modify the transcriptional machinery of BMP-response genes. Whereas the cystine-knot structure of BMPs is a condition precedent for a tight and functional ligand-receptor
contact, the remaining part of BMPs, a heterogeneous N-terminal stretch of around 15-20 amino acids, is not required for this interaction. Due to the high amino acid homology to other BMP members the same is to be expected for MP52.

In order to verify the common "cystine-knot" structure we performed an x-ray crystallography of the BMP subfamily member human MP52. Crystallization of mature MP52 was performed by utilizing the "hanging drop" procedure. Drops of 5 µl protein solution (14 mg/ml dissolved in water) were mixed with 5 µl reservoir solution (100 mM sodium acetate, 3 mM sodium azide and 30% (v/v) isopropanol, pH 5.0) and equilibrated in a hanging drop setup against 1 ml reservoir solution. Crystals appeared in about 3 days. For data collection, a crystal of hexagonal shape measuring 0.4 x 0.4x 0.2 mm3 was used which diffracted to 2.4 Å resolution. Structure solution data were collected at room temperature to 2.4 Å using a 300 mm image plate detector (Mar Research, Hamburg, Germany), mounted on a rotating anode generator model FR571 (Enraf-Nonius, Delft, the Netherlands). The obtained protein structure data were graphically illustrated with the RasWin/RasMol molecular graphics freeware program (R. Sayle

& H. Berstein, 1992-2003). By this method, the molecular structure of the expected "cystine-knot" domain is displayed very clearly (see Figure 1), whereas this technique failed to provide information about the first N-terminal amino acids of mature MP52 (amino acids preceding amino acid 397 (Lys) in SEQ ID NO.1). An obvious explanation for the observed phenomenon is that the position of this stretch is not fixed in the mature MP52 molecule and differs in each crystal. A totally randomized position of the N-terminal end of mature MP52 suggests that it is dispensable for receptor binding and activity.

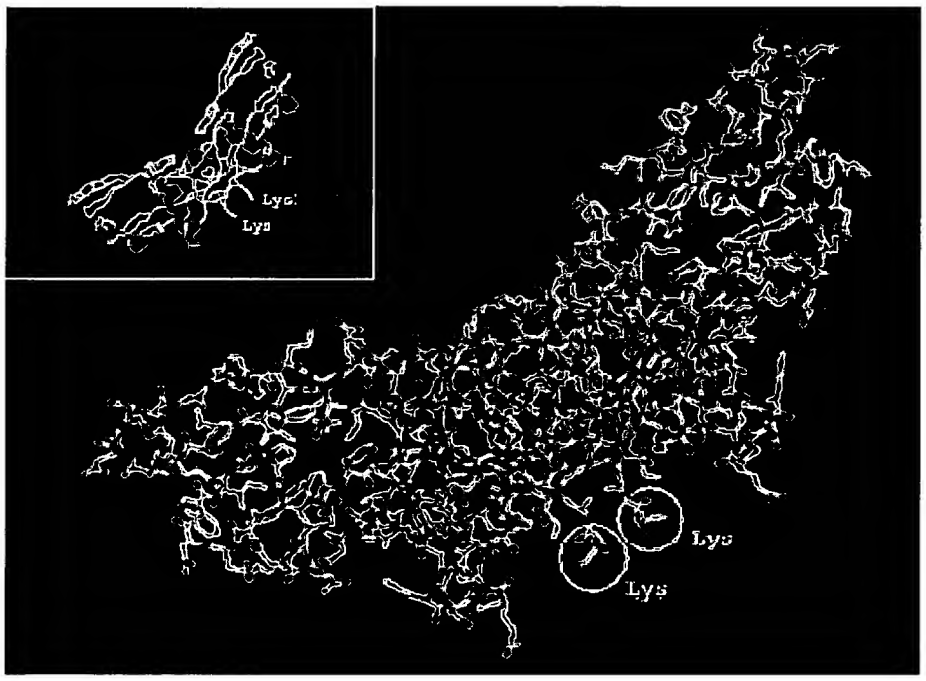


Fig. 1: Structure of dimeric MP52 protein as determined by x-ray crystallography.
Colors:
Atoms: C/gray, N/blue, O/red, S/gold
Ribbons: α-Helix/red, β-sheet/gold, γ-turn: blue
random: gray

In order to verify our suggestion due to the crystallization results and the homology to other BMP members we constructed a fragment version of mature MP52 lacking all N-terminal

amino acids preceding the first cysteine of the cystine-knot domain (Figure 2). The recombinant fragment, MP52-Cys, was expressed in bacteria and tested for biological activity by using the well acknowledged alkaline phosphatase assay (ALP).

CSRKALHVNF KDMGWDDWII APLEYEAFHC EGLCEFPLRS HLEPTNHAVI QTLMNSMDPE STPPTCCVPT RLSPISILFI DSANNVVYKQ YEDMVVESCG CR

Fig. 2: Sequence of the fragment MP52-Cys (amino acids 400-501 of SEQ ID NO.1)

MP52-Cys was cloned and subsequently expressed, refolded and purified according to standard procedures known from mature MP52 (MP52).

For cloning and expression of MP52-Cys, recombinant human MP52 was used as a template to obtain a PCR fragment encoding the cystine-knot-domain of MP52 (Figure 2). Primer sequences were 5' CAT ATG TGC AGT CGG AAG GCA CTG CAT G 3' (MP52-Cys1-NdeI) and 5' AAG CTT GGG ATG TGC CAC CCA GGA AG 3' (MP52-Stop-HindIII). The expected PCR-fragment (about 0.36 kb) is shown in Figure 3 A. This PCR fragment was ligated via Nde I and Hind III restriction sites into predigested prokaryotic expression vector pBPHis. After verification of the nucleic acid sequence this construct was used to transform HMS174 cells. Cells were grown in SOC medium + 3.6 g/l Glucose + 2.03 g/l MgCl2 x 6H2O and induced with IPTG to enhance expression of MP52-Cys. Inclusion bodies harboring recombinant (inactive) monomeric MP52-Cys were obtained by treatment with lysozyme, sonification and subsequent sucrose gradient centrifugation. Protein was released/solubilized from the inclusion bodies (6 M guanidium hydrochloride, 150 mM NaCl, 64 mM DTT, 150 mM Tris-HCl pH 8,0) for 1 hour/ 4°C, followed by centrifugation. The protein was further purified by RP-HPLC (device: Äkta Explorer 100 device, column: Aquapore Octyl ser. no. 186470, eluents: 0.1% TFA/H20 and 0.1 %TFA/90% CH3CN). Refolding to yield active dimeric MP52-Cys was performed for 48 hours at room temperature by use of the glutathione system (1 mM GSSG/ 2

mM GSH/ 33 mM CHAPS) as reducing/oxidizing agent. The successful refolding (dimerization) is shown in Figure 3 B.

Dimeric MP52-Cys was finally separated from inactive monomeric protein by RP-HPLC (columns and conditions as described above) and ultrafiltration (Amicon microcon YM-10) All protein processing steps were confirmed by SDS-Page and western blotting (Primary antibody: polyclonal chicken anti-MP52, secondary antibody: Sigma anti-chicken). Finally purified MP52-Cys is shown in Figure 3 C.

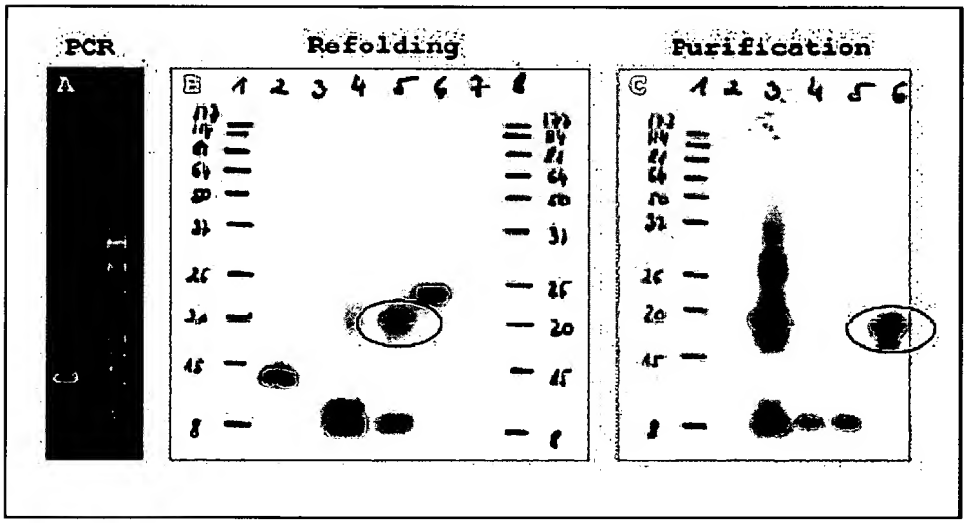
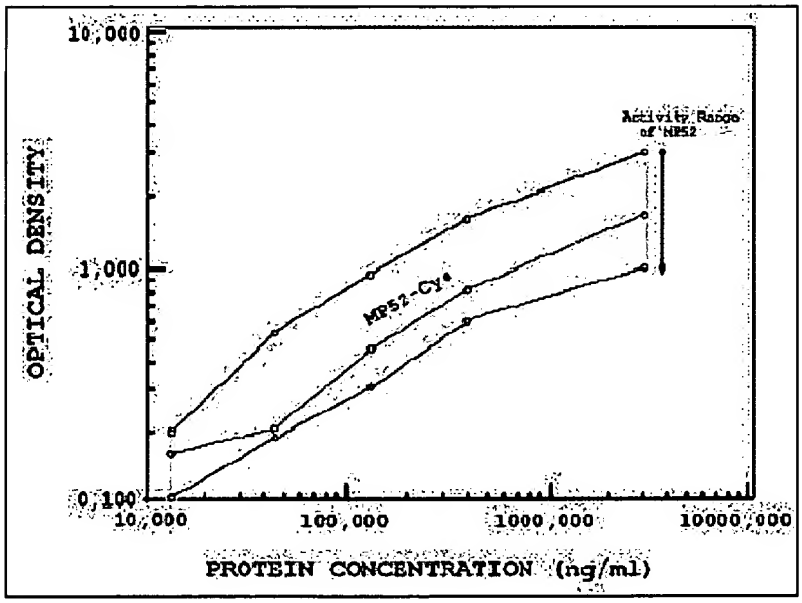


Fig. 3: Amplification, refolding and purification of MP52-Cys.
A) ~ 0.36 kb PCR-fragment encoding MP52-Cys (Molecular weight marker : 100 bp ladder)
B) Refolding of expressed but still monomeric MP52-Cys
Lane 2 + 6: Controls (monomeric and dimeric MP52),
Lane 4: Monomeric MP52-Cys (~10 kD) before refolding
Lane 5: Dimeric MP52-Cys (~20 kD) after refolding (see mark)
C) Purification (RP-HPLC) of refolded MP52-Cys
Lane 3: Mixture of monomeric and dimeric MP52-Cys before RP-HPLC
Lanes 4-6: Different RP-HPLC fractions. Fraction 7 (lane 6) contains purified dimeric MP52-Cys.

The biological activity of MP52-Cys was determined *in vitro* by measurement of increasing alkaline phosphatase (ALP) activity. This established biological activity test measures the

upregulation of alkaline phosphatase and is frequently used to prove the bone inductive capabilities of proteins (Takuwa et al., Am. J. Physiol. 257, E797- E803, 1989). ALP increases the absorbance of the chromogen sodium p-nitrophenyl-phosphate solution at 405nm. Briefly, $5x10^5$ cells of mouse stromal MCHT-1/26 cells were incubated for 3-4 days in 20 ml cell culture medium (alpha-MEM, Penicilline/Streptomycine, 2 mM L-glutamine, 10% FCS) at 37°C, 5% CO2, H2Osaturated. The cells were subsequently washed with PBS (phosphate buffered saline), trypsinated and resuspended in culture medium to a density of 3x104 cells/ml. 150 µl were transferred to each well of a 96 well culture plate and incubated for 24 h at 37° C, 5% CO2, H2O-saturated. After washing with medium the wells were filled with 120µl of new culture medium. 40 µl of different dilutions of MP52-Cys were added, followed by another incubation step for 72 h at 37°C, 5% CO2, H2O-saturated. After washing with PBS, 150 µl of lysis solution (0,2% Nonidet P40, 0,2g MgCl2 x 6H2O, adjusted to 1000 ml with water) was added, followed by 15-18h incubation at 37°C, 5% CO2, H2O-saturated. 50 µl of each well were subsequently transferred to a new 96 well plate. 50 µl of substrate solution (2,5x concentrated diethanolamine substrate buffer + 148g/l PNPP (sodium p-nitrophenyl-phosphate)) was then added to each well and the plates were incubated for another 60 min at 37°C, 5% CO2, H2O-saturated. The ALP-reaction was stopped afterwards with 100 µl of 30g/l NaOH and finally the optical density was measured with an automatic microplate reader at 405 nm under consideration of blank value subtraction. Biological activity of MP52-Cys is calculated as the ratio of ALP activity induced by the test substance (MP52-Cys) to that by the reference standard (MP52).

Induction of alkaline phosphatase *in vitro* clearly proved significant biological activity of MP52-Cys (Figure 4). All OD values (at protein concentrations of 14.8, 44.5, 133.2, 400 and 1200 ng/ml) are within the normal activity range of the reference standard (MP52). According to the specifications of Biopharm's validated ALP-assay and due to the biological nature of the used assay system, a protein is considered as equally active as the reference if its OD values are within 0.5 to 1.5 times that of the reference standard.



The measured biological activity is in the range usually obtained by native MP52. In this initial study MP52-Cys reaches an activity of about 83% of that of highly pure industrial grade MP52. However, the small scale MP52-Cys batch described in this report was produced only in lab-grade quality potentially carrying still some impurities. A biological activity of MP52-Cys up to 100% of native MP52 seems reasonable if the purification method is adjusted to that of the reference standard.

The current results of MP52-Cys prove that a fragment containing only the cystine-knot core of MP52 is well able to bind to its receptor and exhibits the activity of MP52.

3. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

By:

Dr. Jens Pohl

Date:_____